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(54) Title: COLLAGEN PEPTIDES AND USES THEREOF		
(57) Abstract Collagen peptides, fragments and variants are able to interact with and/or bind integrin $\alpha 2\beta 1$, integrin $\alpha 1\beta 1$ and/or platelet receptor GpVI. Such peptides, mimetics thereof and substances that bind them are useful in modulating platelet and other cell function, including aggregation and activation.		

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COLLAGEN PEPTIDES AND USES THEREOF

The present invention relates to peptides, in particular peptides based on collagen sequences, useful in modulating platelet and other cell function, including aggregation and activation. The invention also relates to methods of production of such peptides, their assembly into trimers, and methods of use.

Collagen is the most abundant protein in the human body, where it serves a crucial role in connective tissue, imparting tensile strength to bone, cartilage, skin and tendon, for example. About 20 different collagens are known, the products of discrete genes. Some of these collagens are fibrous in nature, exemplified by type I and type III collagen. Both of these are present in the blood vessel wall. Fibrous collagens are comprised of three α chains assembled as a triple helix, the tropocollagen molecule or collagen monomer. Two gene products, the discrete $\alpha 1(I)$ and $\alpha 2(I)$ chains form type I collagen, which has the structure $[\alpha 1(I)]_2[\alpha 2(I)]_1$. Type III collagen has the trimeric structure $[\alpha 1(III)]_3$. Higher order structure is necessary for fibre formation, where triple helices assemble further to form ordered fibres from arrays of tropocollagen molecules.

For collagen to assemble spontaneously as triple-helices, it is necessary for the primary structure of the α chains to contain triplets of amino acids where the first residue of the triplet is always Glycine (Gly or G), the second is often Proline (Pro or P) and the third is, rather less often, hydroxyproline (Hyp or O, or P*). The collagen sequence is often presented, an oversimplification, as $[GPO]_n$, where $n=330$ or so. Departures from this ideal sequence are sufficiently frequent that GPO triplets actually provide only about 10-12% of types I and III collagen. The corollary of this, of course, is that other residues than P or O occur quite commonly in the second and third positions. The ability of collagen to form triple helices arises from (i) the lack of a bulky side chain in G, so that the single proton substituent takes up little space close to the axis of the helix, (ii) the strained proline ring

structure present in P and O allowing particular bends to occur in the polypeptide backbone of collagen so that triple-helical structure can be adopted, and (iii) intra-chain hydrogen bonding involving the hydroxyl group of O adding to the stability of the helix.

Cells are able to interact with collagen, either directly or indirectly, through the agency of specific cell surface proteins usually known as receptors. Often these are glycoproteins (Gps). Examples of direct interaction would be, in platelets, binding of collagen to the integrin $\alpha 2\beta 1$, (also known as GpIa-IIa or VLA₂ or CD49b/CD29) or binding to the uncharacterised receptor, GpVI (Kehrel, 1995; Sixma et al, 1995, Sixma et al 1997; Moroi and Jung, 1997). Examples of indirect interaction include the use of accessory molecules, e.g. von Willebrand factor, to form a bridge or crosslink between a specific domain of the collagen molecule and GpIB.

Many different cell types interact with collagen in these ways. Cell-collagen interactions are necessary for processes including wound healing, tumour metastasis, angiogenesis and neovascularisation, also (of particular interest) the activation of platelets leading to platelet aggregation which is the acute cause of thrombosis. Collagen may also support cell migration, including of vascular smooth muscle cells from the media of the artery to the intima, contributing to the development of atheroma, thickening of the arterial wall in atheroma. Whilst cells are induced to migrate by chemoattractant stimuli such as growth factors, they need physical adhesion to an underlying substrate to allow them to move. The integrins may provide the mechanical support, therefore, for cell motility.

The present invention is based in part on experimental work concerning triple-helicity as a pre-requisite for cell-collagen interaction. Collagen can be denatured (melted) by heating above a critical temperature, typically 40°C or higher, and when suitable precautions are taken to avoid the re-adoption of triple-helical structure, many cells do not recognise this denatured collagen, more commonly known as gelatin. Unless denatured collagen is allowed to re-form tropocollagen molecules, and for these to re-assemble as fibres, it does not

interact with the platelet surface to cause platelet activation.

Collagen receptors may be of several types, although the molecular architecture of some of these is not fully defined. The platelet receptor GpVI, for example, is identified by its
5 molecular weight, (~65 kDa), by auto-antibodies possessed by a very few patients identified to date in Japan (Sugiyama et al 1993), by the capacity to bind a particular snake venom known as convulxin (Polgar et al., 1997) and, now, by means of work using GpVI-deficient platelets, by the capacity to bind synthetic
10 peptides of the invention, such as a GPO-based polymer known as "Collagen-Related Peptide" or "CRP" (Morton et al., 1995; Gibbins et al., 1997; Kehrel et al., 1998). The sequence of GpVI is not known, nor is its membrane topology, but it is defined in Moroi and Jung, 1997. Another platelet collagen
15 receptor, CD36 or GpIV, is sequenced and has a single trans-membrane domain (Greenwalt et al., 1992; Platt & Gordon, 1998). There is indication that CD36 is involved in the adhesion to other tissues of malarial-infected red cells (Ockenhouse et al., 1989). CD36-binding motifs in the collagens are not known.

20 The integrin $\alpha 2\beta 1$ is a better-defined collagen receptor. It consists of two non-identical subunits, $\alpha 2$ and $\beta 1$, members of the integrin family each with a single trans-membrane domain, and $\alpha 2\beta 1$ is known to bind to collagen via a specialised region of the $\alpha 2$ -subunit, known as the I-domain, which bears sequence
25 homology to the I-domain of von Willebrand factor. The integrins are widely-distributed in different cells, and cell-collagen interaction at large may often be mediated directly by $\beta 1$ integrins, either $\alpha 2\beta 1$ or $\alpha 1\beta 1$ (Hynes, 1992).

It has long been known that some integrins recognise a
30 particular arrangement of amino acids in their ligands containing the motif RGD. This mediates the attachment of several proteins, for example fibronectin and fibrinogen, to their integrin receptors. Peptide antagonists of these receptors have been developed which employ either this RGD motif
35 or a derivative of it.

Staatz et al., 1991, *J. Biol. Chem.* 266, 7363-7367, ascribed the site of interaction of collagen with the integrin $\alpha 2\beta 1$ to the sequence DGEA, residues 435-438 of the $\alpha 1(I)$ chain.

It is known, however, that there are several $\alpha 2\beta 1$ recognition sites within collagens. This knowledge arises from the use of collagen fragments derived from purified α chains, hydrolysed into specific and reproducible peptides using cyanogen bromide (CB) which cleaves specifically at methionine residues in the α -chain. Some of these CB peptides are quite long, perhaps more than 100 amino acids, and can be renatured to form defined triple-helical fragments of the parent collagen molecules. They are known by their order of elution from the purification procedure, as for example $\alpha 1(I)CB3$ - the third CB peptide from the $\alpha 1$ -chain of type I collagen. Some of these CB peptides, renatured and in triple-helical form, bind to platelets, whereas others do not. The use of monoclonal or other specific antibodies directed against particular receptors allows the identification of the platelet collagen receptors supporting this interaction. This type of experiment has revealed that some CB peptides support binding of platelets via $\alpha 2\beta 1$, whereas others are inactive (Morton et al., 1989; Morton et al., 1994).

Further, synthesis of these active CB peptides as a series of overlapping short peptides has allowed particular sequences or regions of the parent collagen molecule to be identified as contributing to the recognition of collagen by $\alpha 2\beta 1$. Thus, a particular sequence from Type III collagen, located within $\alpha 1(III)CB4$, GGPOGPR representing residues 522-528 of the Type III α -chain, has been identified as contributing to an integrin $\alpha 2\beta 1$ -recognition site in Type III collagen (Morton et al., 1997).

This sequence is not long enough, nor has it sufficient GPO content to adopt a triple-helical conformation spontaneously. To achieve this, it is synthesised with several GPO triplets at either end of the sequence in question. GPP triplets will serve the same purpose. The C-terminus may be covalently linked using N-linked aminohexanoate residues coupled to a lysyl-lysyl group. The incorporation of a cysteine or other reactive residue at the N-terminus or C-terminus or both of each of the three component peptide chains allows subsequent crosslinking of the triple-helical structures (Morton et al., 1995).

In accordance with the present invention various peptides have been synthesized and the $\alpha 2\beta 1$ -recognition site of type I collagen, located within $\alpha 1(I)CB3$, has been identified as residing within residues 502-516 of the $\alpha 1(I)$ chain. Peptide fragments of $\alpha 1(I)CB3$ have been synthesized, as a series of overlapping peptides, incorporating them within GPO or GPP motifs as above, but, with one exception, without the use of C-terminal covalent crosslinking using hexanoic acid as outlined above. In addition, certain modifications of sequence have been performed, deleting certain amino acid triplets from the 15-residue sequence defined above, and substituting specific amino acids including A for E as shown. The peptides have been found to have a melting temperature sufficiently high to use for experimental purposes.

Peptides so produced comprising GPO and GPP as a means of stabilising the triple-helix are shown in Table 1.

The $\alpha 2\beta 1$ recognition sequence embraced by these peptides, identified as being contained by the collagen-derived sequence from the overlap region of peptides 5 and 6, re-synthesised as Table 1 peptide 5/6, was also synthesised within GPP N-terminal and C-terminal sequences, as an alternative means of stabilising the triple helix. This peptide, lacking GPO sequences, does not react with GpVI and is thus specific for $\alpha 2\beta 1$.

In accordance with one aspect of the present invention there is provided a peptide fragment of collagen consisting of the amino acid sequence GFOGERGVEGPOGPA (SEQ ID NO. 1), or a fragment thereof which retains ability to interact with and/or bind the $\alpha 2\beta 1$ receptor.

Furthermore, the present invention also provides a peptide of the sequence GFPGERGVEGPPGPA (SEQ ID NO. 2), that is the collagen fragment of the first aspect of the invention in which Proline (P) is substituted for Hydroxyproline (O). As demonstrated experimentally herein, such a modified fragment surprisingly retains good binding activity for the $\alpha 2$ I-domain of the $\alpha 2\beta 1$ integrin receptor.

Also provided are peptides which are sequence variants of these two peptides SEQ ID NO. 1 and SEQ ID NO. 2, which are made that are successively shorter by a single amino acid or more, to

locate the minimum sequence recognising $\alpha 2\beta 1$. One such peptide has the active sequence, GFOGER, SEQ ID NO.3.

Also provided are peptides which are sequence variants of one of these peptides SEQ ID NO.1, SEQ ID NO. 2 and SEQ ID NO.3, retaining ability to bind $\alpha 2\beta 1$, and having at least 10/15 amino acid identity with SEQ ID NO. 1 or SEQ ID NO. 2, preferably at least 11/15 amino acid identity, more preferably 12/15, 13/15 or 14/15, or having at least 3/6 amino acid identity, or more preferably 4/6 or 5/6 amino acid identity with SEQ ID NO. 3. Preferably a peptide according to such an aspect retains each of the phenylalanine (F), the glutamyl (E), and the arginyl (R) residues within the GFOGER motif, assuming ability to bind $\alpha 2\beta 1$ is desired. Binding activity may be retained if the Hydroxyproline (O) is substituted with proline (P), the E is substituted with Aspartate (D), or the R is substituted with Lysine (K) and any other substitution which does not abolish binding activity may be made, the person skilled in the art easily being able to test this. Where it is desired to reduce or abolish such ability one or other of these critical residues may be replaced with another amino acid, such as A (as experimentally exemplified), or residues such as His, Lys or Arg, particularly Tryp, Tyr or Phe. Such peptides serve as structurally-inactive analogues, useful for example in in vitro adhesion studies.

Non-naturally occurring peptides and polypeptides including such a collagen fragment are also provided as aspects of the present invention, particularly wherein the collagen fragment or variant thereof is fused to one or more non-collagen sequences.

Thus, for example, the invention also includes derivatives of the peptides, including the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule, and/or a targeting molecule such as an antibody or binding fragment thereof or other ligand. Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art.

Peptides according to various aspects of the present invention may be able to bind the $\alpha 2$ I-domain of the $\alpha 2\beta 1$

integrin receptor separate or in isolation from the remainder of the integrin, or in the complete heteromer. Peptides may be assessed for ability to bind using complete $\alpha 2\beta 1$, the $\alpha 2$ I-domain, or a fragment or variant thereof able to bind collagen and/or bind the GFOGERGVEGPOGPA motif or GFOGER of the invention.

According to the present invention there is provided a peptide including GPP repeats and a collagen fragment, or variant or derivative thereof as discussed, preferably a collagen fragment or variant able (at least in a trimer) to interact with and/or bind the $\alpha 2\beta 1$ collagen receptor. Such a peptide will form a trimer under appropriate conditions, but will not bind and/or stimulate the collagen receptor GpVI. The peptide is non-naturally occurring, i.e. it is one not found in nature. Generally, GPP repeats are included so as to flank said collagen fragment or variant, and may be at or adjacent to the termini of the peptide.

The collagen fragment or variant able to interact with and/or bind the $\alpha 2\beta 1$ collagen receptor may include or consist of the GGPOGPR fragment (Morton et al. 1997) or a fragment or variant in accordance with other aspects of the invention as disclosed herein, including in the discussion above (e.g. GFOGERGVEGPOGPA or GFPGERGVEGPPGPA or GFOGER).

This provides for trimers of the peptide which are able to interact with the $\alpha 2\beta 1$ collagen receptor but not the GpVI collagen receptor.

Since peptides of the invention, exemplified by the GFOGER motif of SEQ ID NO. 3, are also recognised by the $\alpha 1$ integrin I-domain, they may be used interact with and modulate activity of integrin $\alpha 1\beta 1$.

In a further aspect, the present invention provides a peptide including GPO repeats and a collagen fragment or variant or derivative thereof as disclosed, preferably a collagen fragment or variant able (at least in a trimer) to interact with and/or bind the $\alpha 2\beta 1$ collagen receptor. Such a peptide will form a trimer under appropriate conditions, and will bind and/or stimulate the collagen receptor GpVI. The peptide is non-naturally occurring, i.e. it is one not found in nature.

Generally, GPO repeats are included so as to flank said collagen fragment or variant, and may be at or adjacent to the termini of the peptide.

5 The collagen fragment or variant able to interact with and/or bind the $\alpha 2\beta 1$ collagen receptor may include or consist of the GGPOGPR (Morton et al. 1997) but is most preferably a fragment or variant in accordance with other aspects of the invention as disclosed herein, including in the discussion above.

10 This provides for trimers of the peptide which are able to interact with the $\alpha 2\beta 1$ collagen receptor and are also able to interact with the GpVI collagen receptor.

A molecule in accordance with the present invention may include GPP and/or GPO units.

15 Furthermore, triple helical conformation may be supported by repetitive GPY motifs at each end of the specified sequence, where, in addition to the possibilities present by P and/or O, any amino acid which preserves triple-helical stability, e.g. A or R, may be used.

20 Peptides of the invention need not include any hexanoic acid cross-linking for trimerization (such as the lysyl-lysyl amino hexanoate cross-linking illustrated experimentally below).

A peptide in accordance with an aspect of the present invention may include one or more heterologous amino acids
25 joined to the collagen fragment, in addition to the optional GPP or GPO repeats. By "heterologous" is meant not occurring in the relevant collagen joined by a peptide bond without intervening amino acids to the relevant collagen fragment, that is to say usually a chain of amino acids which is not found naturally
30 joined to the collagen fragment at the position of fusion in the peptide of the invention. Usually where heterologous amino acids are included, the contiguous sequence of amino acids does not occur within collagen, and may be 5 or more, preferably 10 or more, more preferably 15 or more, 20 or more or 30 or more
35 amino acids with a sequence which does not occur contiguously in collagen.

Peptides according to different aspects of the present invention tend to be short, and may be about 60 amino acids in

length or less, preferably about 50 amino acids in length or less, preferably about 40 amino acids in length or less, preferably about 35 amino acids in length or less, preferably about 30 amino acids in length, or less, preferably about 25 amino acids or less, preferably about 20 amino acids or less, preferably about 15 amino acids or less, preferably about 10 amino acids or less. Peptides may be 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-50 or 50-60 amino acids in length. Where GPP and/or GPO repeats are included the number of triplet repeats may be about 3-5, possibly 2, preferably 3, 4 or 5. Greater than 5 may be included (perhaps less than about 10), though may not be necessary since 3, 4 or 5 repeats are stable as a triple helix at room temperature.

Peptides according to different aspects of the present invention may be homotrimeric as described above, where all three chains have identical sequence, but may also be heterotrimeric in nature, that is at least one of the chains is different from the others, and two but not three chains may be identical in sequence. In one embodiment, one or two but not three chains may incorporate receptor-specific recognition motifs, such as SEQ ID NO. 3, GFOGER, specific for $\alpha 2\beta 1$ or $\alpha 1\beta 1$, or GPO motifs specific for GpVI. In such peptides it may be necessary to include covalent crosslinking, preferably at the C-terminus, using suitable means. Procedures suitable for assembling heterotrimers are established in the art, e.g. M. Goodman et al, (1996). A template-induced incipient collagen-like triple-helical structure. J. Am. Chem. Soc. 118, 5156-5157; J. Ottl and L. Moroder, (1999). Disulfide-bridged heterotrimeric collagen peptides containing the collagenase cleavage site of collagen type I. Synthesis and conformational properties. J. Am. Chem. Soc. 121, 653-661. J. Ottl and L. Moroder, (1999). A new strategy for regioselective interstrand disulfide bridging of multiple cysteine peptides. Tetrahedron Lett. 40, 1487-1490.

A peptide according to the present invention may be provided as a fusion with a non-collagen peptide or polypeptide or polypeptide domain. Where it is desired to take advantage of the trimerizing properties of peptides of the invention, any fusion will need to be such as not to interfere with the

trimerization. Multiple specificities may be presented in a single molecule, e.g. for crosslinking receptors to $\alpha 2\beta 1$ and/or GpVI, allowing for examination of interactions between receptors and of intracellular events resulting from such heterogeneous interactions, also for such biological response to be harnessed in therapeutic and/or diagnostic contexts.

The present invention provides for the incorporation of multiple copies of receptor-reactive sequences, to induce homogeneous crosslinking of the specified or other receptors. Thus, peptides containing two or more copies of the sequence GFOGER, spaced at suitable intervals, for example by intervening GPP triplets, up to 10 triplets, may be used for crosslinking of two copies of the receptor $\alpha 2\beta 1$. Similarly, sequences specific for other collagen receptors, such as GPO which is specific for the receptor GpVI, may be used for crosslinking of GpVI. These peptides may be maintained in triple-helical form by the presence of GPP flanking triplets as disclosed.

In a further aspect the present invention provides peptide trimers including one or more peptides according to the present invention, preferably consisting of three peptides according to the invention.

A still further aspect provides a method of making a peptide trimer, the method including providing peptides of the invention and causing or allowing (under appropriate conditions) the peptides to associate to form a trimer.

Trimerization may be followed by isolation of trimers, e.g. for subsequent use and/or manipulation.

Recognition motifs contained within flanking sequences which do not permit the adoption of triple helical structure, for example polymers of GAP, may not support the ability of the sequence to interact with the receptor. Such peptides may form important, inactive control preparations in assays of biological activity. This is exemplified in the Results section.

As an alternative to trimerization, peptides in accordance with the present invention may be cyclized. This reduces the degrees of freedom of a peptide substantially, so that it may interact with higher affinity with suitably-configured targets, such as receptors. Chemistry for achieving this is available in

the art, e.g. on commercially available solid-phase peptide synthesizers. Peptides may be provided as cyclic dendrimers, species bearing the desired peptides as loops, typically of 5 or so at each end of a short spacer sequence (Shao and Tam, 1995).

5 Peptides, particular in trimerized or cyclized form, in accordance with aspects of the present invention, may be used in influencing cell adhesion to collagen and/or other cell types, particularly adhesion of platelets, smooth muscle cells, tumour cells or vascular endothelial cells. They may be used to affect
10 activation of cells, such as platelets, modulating, particularly inhibiting or blocking, activation. This may be in a therapeutic context, e.g. to treat or prevent thrombosis.

 Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be
15 readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984),
20 in M. Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); in J. H. Jones, The Chemical Synthesis of Peptides. Oxford University Press, Oxford 1991; in Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California , in G. A. Grant, (Ed.) Synthetic Peptides, A
25 User's Guide. W. H. Freeman & Co., New York 1992, E. Atherton and R.C. Sheppard, Solid Phase Peptide Synthesis, A Practical Approach. IRL Press 1989 and in G.B. Fields, (Ed.) Solid-Phase Peptide Synthesis (Methods in Enzymology Vol. 289). Academic Press, New York and London 1997), or they may be prepared in
30 solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the
35 respective carbonic or sulfonic acid or a reactive derivative thereof.

 A suitable technique for providing peptides according to the invention is exemplified in more detail in the experimental

section below.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system. This is particularly applicable for peptides not including O, such as those including GPP repeats and no GPO repeats and those including or based on SEQ ID NO. 2, rather than SEQ ID NO. 1, although production of GPO-containing peptides may be achieved for example by co-expression of an appropriate hydroxylase, as has been done with lysyl residues (Nokelainen et al., 1998). For peptides containing Pro residues to be post-translationally converted by hydroxylation to Hyp (O), prolyl-hydroxylase may be co-expressed.

Accordingly the present invention also provides in various aspects nucleic acid encoding the polypeptides and peptides of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, except possibly one or more regulatory sequence(s) for expression. Nucleic acid in accordance with the present invention may be provided as part of a recombinant vector.

Nucleic acid sequences encoding a polypeptide or peptide in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Current Protocols in Molecular Biology, John Wiley and Sons, 1992).

In order to obtain expression of the nucleic acid sequences, the sequences can be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide produced in the host cell is secreted from the cell. Polypeptide can

then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium. Prokaryotic and
5 eukaryotic cells are used for this purpose in the art, including strains of *E. coli*, yeast, and eukaryotic cells such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or peptide (as disclosed), the method
10 including expression from nucleic acid encoding the polypeptide or peptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide.
15 Polypeptides and peptides may also be expressed in *in vitro* systems, such as reticulocyte lysate.

As noted, methods of making peptides by chemical synthesis are also encompassed by the present invention.

Thus, a further aspect of the present invention provides a
20 host cell containing heterologous nucleic acid as disclosed herein. The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard
25 techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The
30 introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection
35 and transduction using retrovirus or other virus, e.g. vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an

alternative, direct injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing nucleic acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide (or peptide) is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide or peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Peptides and polypeptides in accordance with the present invention may be used in assays for molecules which affect interaction of collagen with $\alpha 2\beta 1$ receptor and/or GpVI receptor, particularly molecules which are non-peptidyl mimetics of peptides such as those disclosed herein able to bind $\alpha 2\beta 1$ and/or GpVI.

An assay method in accordance with the present invention may involve bringing into contact a peptide of the invention and either or both of the $\alpha 2\beta 1$ and GpVI receptors, along with a test substance, under conditions wherein in the absence of the test substance being an inhibitor of interaction between the peptide and the receptor the peptide and receptor interact, and determining interaction. Where it is desired to provide a substance which is able to enhance interaction between a peptide or the invention and the relevant receptor, an assay may be designed in which the peptide and receptor are provided under conditions in which they do not interact unless a test substance is able to sufficiently enhance interaction.

The precise format of an assay of the invention may be varied by those of skill in the art using routine skill and knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support. Suitable detectable labels, especially for peptidyl substances include ³⁵S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein containing an epitope which can be labelled with an antibody.

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis. Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

A two-hybrid assay may be designed in accordance with standard practice.

Peptides and other molecules may be assessed for ability to bind $\alpha 2\beta 1$ and/or GpVI collagen receptors, modulate, particularly inhibit or block, platelet activation, or cellular adhesion (e.g. of HT 1080 cells), affect cell migration, for example of vascular smooth muscle cells, tumour cells or vascular endothelial cells or to regulate cell proliferation or cell survival.

Assays may involve test substances which block the action of native collagen fibres, in aggregation or adhesion or other tests of cell function. Substances may be used or found which

displace cells from an underlying native collagen substrate. Alternatively, peptides may form an adhesive substrate, with test substances being applied to test efficacy in displacing cells or receptors which bind, or in affecting one or more cellular processes such as cell migration, proliferation or survival.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 nM to 100 μ M or more concentrations of putative inhibitor compound may be used, for example from 0.1 to 50 μ M, particularly 3nM to 30 μ M, such as about 10 μ M. Greater concentrations may be used when a peptide is the test substance. Even a molecule with weak binding may be a useful lead compound for further investigation and development.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction.

Antibodies may be obtained using techniques which are standard in the art.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which tests positive.

The substance or agent may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals, e.g. for any of the purposes discussed elsewhere herein.

As noted, the agent may be peptidyl, e.g. a peptide which includes a sequence as recited above, or may be a functional

analogue of such a peptide.

As used herein, the expression "functional analogue" relates to peptide variants or organic compounds having the same functional activity as the peptide in question, which may
5 interfere with the binding between collagen and the relevant receptor. Examples of such analogues include chemical compounds which are modelled to resemble the three dimensional structure of the relevant collagen fragment in the contact area, and in particular the arrangement of the key amino acid residues as
10 they appear in collagen.

In a further aspect, the present invention provides the use of the above substances in methods of designing or screening for mimetics of the substances.

Accordingly, the present invention provides a method of
15 designing mimetics of a peptide or other molecule of the invention, said method including:

(i) analysing a substance having the relevant biological activity (in particular ability to interact with $\alpha 1\beta 1$, $\alpha 2\beta 1$ or GpVI receptor or enhance or interfere with collagen interaction
20 with either receptor) to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

25 Suitable modelling techniques are known in the art. This includes the design of so-called "mimetics" which involves the study of the functional interactions of fluorogenic derivatives of target receptors, e.g. $\alpha 1\beta 1$, $\alpha 2\beta 1$ or GpVI, with their respective recognition motifs and the design of compounds which
30 contain functional groups arranged in such a manner that they could reproduce those interactions.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be
35 desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides may not be well suited as active agents for oral compositions as they tend to be quickly degraded

by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property.
5 Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide,
10 e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g.
15 stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other
20 techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to
25 take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to
30 synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further
35 optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

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what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

5 Mimetics of this type together with their use in therapy form a further aspect of the invention.

The present invention further provides the use of a peptide which includes a sequence as disclosed, or a derivative, active portion, analogue, variant or mimetic, thereof able to interact with the $\alpha 2\beta 1$ collagen receptor and/or the GpVI collagen
10 receptor, in screening for a substance able to modulate interaction between collagen and such a receptor.

Generally, such a substance, e.g. peptide, mimetic, inhibitor, according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This
15 may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients. As
20 noted below, a composition according to the present invention may include in addition to an inhibitor compound as disclosed, one or more other molecules of therapeutic use.

The present invention extends in various aspects not only to peptides and other substances identified as disclosed, but
25 also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for a purpose discussed elsewhere herein, which may include preventative treatment, use of such a substance in manufacture
30 of a composition for administration, e.g. for a purpose discussed elsewhere herein, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

35 A pharmaceutically useful compound according to the present invention that is to be given to an individual, is preferably administered in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although

prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier formulations.

Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition,

5 Osol, A. (ed), 1980.

The agent may be administered in a localised manner to a desired site or may be delivered in a manner in which it targets particular cells or tissues.

A composition may be administered alone or in combination
10 with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

A polypeptide, peptide or other substance as disclosed herein, or a nucleic acid molecule encoding a peptidyl such molecule, may be provided in a kit, e.g. sealed in a suitable
15 container which protects its contents from the external environment. Such a kit may include instructions for use.

Peptides and other substances according to the present invention as disclosed herein may be directed by virtue of $\alpha 2\beta 1$ - and/or GpVI-recognition motifs towards particular cellular
20 receptors for collagen. The following applications are provided as further aspects of the present invention:

1. Inhibition of platelet activation, e.g. in controlling coronary thrombosis or related cardiovascular disease.

2. Inhibition of cell migration, e.g. in any of a number
25 of different pathologies, including metastasis and atherogenesis. These same peptides would find similar application in experimental studies *in vitro*.

3. Diagnostic testing, e.g. in labelling specific collagen receptors. Peptides may be covalently labelled with an
30 indicator such as a fluorescent dye, e.g. fluorescein or rhodamine, or a radiochemical label, e.g. ^{125}I , or biotin, allowing subsequent combination with labelled streptavidin. Reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of
35 reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding peptide or polypeptide and reporter

molecule. One favoured mode is by covalent linkage of a peptide with an individual fluorochrome, phosphor or laser dye with spectrally isolated absorption or emission characteristics.

Suitable fluorochromes include fluorescein, rhodamine, phycoerythrin and Texas Red. Suitable chromogenic dyes include 5 diaminobenzidine. Other reporters include macromolecular colloidal particles or particulate material such as latex beads that are coloured, magnetic or paramagnetic, and biologically or chemically active agents that can directly or indirectly cause 10 detectable signals to be visually observed, electronically detected or otherwise recorded. These molecules may be enzymes which catalyse reactions that develop or change colours or cause changes in electrical properties, for example. They may be 15 molecularly excitable, such that electronic transitions between energy states result in characteristic spectral absorptions or emissions. They may include chemical entities used in conjunction with biosensors. Biotin/avidin or biotin/streptavidin and alkaline phosphatase detection systems may be employed.

20 The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Methods may be performed *in vitro* on samples of cells 25 and/or tissues removed from the body, or *in vivo*.

4. Raising or obtaining antibodies against defined sequences within the collagens. These antibodies may be used in research as a means of locating receptor binding domains of the collagens.

30 Antibodies which are specific for a target of interest may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunizing a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof, or a cell or virus which 35 expresses the protein or fragment. Immunization with DNA encoding a target polypeptide is also possible (see for example Wolff, et al. *Science* 247: 1465-1468 (1990); Tang, et al. *Nature* 356: 152-154 (1992); Ulmer J B, et al. *Science* 259: 1745-1749

(1993)). Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al, 1992, Nature 357: 80-82).

The production of monoclonal antibodies is well established in the art. Monoclonal antibodies can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. A hybridoma producing a monoclonal antibody may be subject to genetic mutation or other changes, which may or may not alter the binding specificity of antibodies produced.

As an alternative or supplement to immunizing a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with the target, or may be one constructed using sequences obtained from an organism which has been exposed to the antigen of interest (or a fragment thereof).

Antibodies may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any specific binding substance having an binding domain with the required specificity. Thus this covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including any polypeptide comprising an immunoglobulin binding domain, whether natural or synthetic. Chimaeric molecules comprising an immunoglobulin binding domain, or equivalent, fused to another

polypeptide are therefore included. Cloning and expression of chimaeric antibodies are described in EP-A-0120694 and EP-A-0125023.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Example binding fragments are (i) the Fab fragment consisting of VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single antibody; (iv) the dAb fragment (Ward, E.S. et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (v) isolated CDR regions; (vi) F(ab')₂ fragments, a bivalent fragment comprising two linked Fab fragments (vii) single chain Fv molecules (scFv), wherein a VH domain and a VL domain are linked by a peptide linker which allows the two domains to associate to form an antigen binding site (Bird et al, *Science*, 242, 423-426, 1988; Huston et al, *PNAS USA*, 85, 5879-5883, 1988); (viii) bispecific single chain Fv dimers (PCT/US92/09965) and (ix) "diabodies", multivalent or multispecific fragments constructed by gene fusion (WO94/13804; P. Holliger et al *Proc. Natl. Acad. Sci. USA* 90 6444-6448, 1993).

5. Raising and identifying antibodies specific for collagen receptors. Peptides according to the invention may be used to identify receptor-specific antibodies, for instance in bacteriophages libraries (which may conveniently be in a scFv format). This may be by displacement or other means of selection using purified receptor preparations, e.g. of $\alpha 2\beta 1$, or of the I-domain of the $\alpha 1$ subunit of $\alpha 1\beta 1$, the I-domain of the $\alpha 2$ subunit of $\alpha 2\beta 1$, or from cells expressing collagen receptors such as platelets or HT1080 cells, expressing $\alpha 2\beta 1$, or from other cells such as megakaryocytes or platelets which express GpVI. In this way, antibodies specific for such collagen receptors may be identified. These may have similar properties and applications as peptides in accordance with the present invention. Thus the peptides provide a route to the production of another set of materials (antibodies - e.g. scFvs) which may offer certain advantages, such as protection from proteolysis, when used therapeutically, compared with peptides of the invention.

6. Locating domains within collagens which interact with other collagen receptor molecules, e.g. CD36 (GpIV). These interactions may present therapeutic targets themselves.

7. Locating domains within collagens which interact with other, non-receptor molecules, e.g. other extracellular proteins such as fibronectin or von Willebrand factor. These interactions may present therapeutic targets themselves. A further example of this might be the binding site for the glycosaminoglycan, decorin, which binds to collagen in the vascular subendothelium, providing a locus for lipoprotein accumulation (Pentikainen, et al., 1997) which may be involved in the development of atheroma.

Aspects and embodiments of the present invention will now be illustrated by reference to the following experimental exemplification and support, and the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art in view of the present disclosure. The term "comprise" as used herein has the meaning of "include", i.e. permitting the presence of one or more additional components. All documents mentioned anywhere herein are incorporated by reference.

Figure 1 shows the melting curve of peptide 6, plotting change in optical rotation ($^{\circ}$) against temperature ($^{\circ}\text{C}$).

Figure 2 illustrates the binding of type I collagen and various $\alpha 1(\text{I})$ CB3 peptides (Peptides 1 to 4) to $\alpha 1\beta 2$ integrin, the chart showing A_{450} at various concentrations of substrate ($\mu\text{g/ml}$). Squares represent type 1 collagen, diamonds peptide 1, circles peptide 2, triangles peptide 3, crosses peptide 4.

Figure 3 illustrates the binding of type I collagen and various $\alpha 1(\text{I})$ CB3 peptides (Peptides 5 to 7) to $\alpha 1\beta 2$ integrin, the chart showing A_{450} at various concentrations of substrate ($\mu\text{g/ml}$). Squares represent type 1 collagen, diamonds peptide 5, circles peptide 6, triangles peptide 7.

Figure 4 shows binding of collagen, Peptide 5/6 and Peptide 5/6GEA and 5/6GAR to $\alpha 1\beta 2$ integrin in the absence and presence of Mg^{2+} the chart showing A_{450} .

Figure 5 shows binding of collagen and Peptides Des-GVE, and Des-GPO to $\alpha 2$ I-domain in the absence and presence of EDTA,

and the inhibition of binding by the anti- $\alpha 2$ antibody, 6F1, the chart showing A_{450} at various concentrations of substrate ($\mu\text{g/ml}$).

Figure 6 shows, as for Figure 5, binding of collagen and Peptides Des-GPA and Des-GFO to $\alpha 2$ I-domain in the absence and presence of EDTA, and in the presence of 6F1, the chart showing A_{450} .

Figure 7 shows binding of collagen and Peptides DesGVE and GFOGDR and to $\alpha 2\beta 1$ in the absence and presence of EDTA, and the effect of 6F1, the chart showing A_{450} .

Figure 8 shows the binding of Peptides Des-GVE and GFOGDR to the $\alpha 1$ I-domain, and inhibition of binding by an anti- $\alpha 1$ antibody, AB, the chart showing A_{450} .

Figure 9 shows binding of collagen and Peptides Des-GVE-GPP, GFOGEK and MADE42 to integrin $\alpha 2$ I-domain, in the presence and absence of Mg^{2+} and the anti- $\alpha 2$, 5E8, the chart showing A_{450} .

Figure 10 shows the ability of the peptide Des-GVE-GPP to inhibit platelet adhesion to collagens type I and type IV, the chart showing A_{405} . Squares represent collagen type 1, diamonds collagen type IV.

Figure 11 shows the ability of the peptide Des-GVE-GPP to inhibit binding of $\alpha 1$ and $\alpha 2$ I-domains to type I collagen, the chart showing A_{450} . Squares represent $\alpha 1$ I-domain, diamonds $\alpha 2$ I-domain.

Figure 12 shows the ability of the peptide Des-GVE-GPP to inhibit binding of $\alpha 2$ I-domain to type I collagen, whereas DesGVE-GAP is inactive, the chart showing A_{450} . Squares represent Des-GVE-GPP, diamonds Des-GVE-GAP.

Figure 13 shows the adhesion of platelets to the peptides CRP, GPP10, AcGPO2, AcGPO4 and to collagen, and the effect of the presence of Mg^{2+} or other divalent cations, the chart showing A_{405} .

Figure 14 shows the adhesion of platelets to the peptides AcGPO4, CRP, and to collagen, and the effect of the presence of Mg^{2+} , the anti- $\alpha 2$, 6F1, or GpVI-specific Fab fragments, the chart showing A_{405} .

Figure 15 shows binding of collagen and Peptides Des-E-GPP, 56Hyp and GPP-56 to integrin $\alpha 2$ I-domain in the presence and

absence of Mg^{2+} , the chart showing A_{450} .

EXPERIMENTAL

Peptide fragments of $\alpha 1(I)CB3$ were synthesized, as a series
5 of overlapping peptides, incorporating them within GPO or GPP
motifs as above, but, with one exception, without the use of C-
terminal covalent crosslinking using hexanoic acid as outlined
above. In addition, certain modifications of sequence have been
performed, substituting A for E as shown. The peptides have
10 been found to have a melting temperature sufficiently high to
use for experimental purposes.

Peptides so produced comprising GPO and GPP as a means of
stabilising the triple-helix are shown in Table 1.

Previously, alignment of constituent chains was guaranteed
15 by synthesizing them on a template comprising the lysyl-lysyl
aminohexanoate structure, thought to be needed for peptides with
a low GPO content. However, it has now been found that
alignment may be self-determining, representing the minimum free
energy state of the associated peptides, and peptides may
20 associate properly without hexanoic acid cross-linking.

(GPP) $_n$ with sequence GCP(GPP) $_{10}$ GCPG was found to have a
melting temperature ($T_{m1/2}$) of 41°C.

25 (GPA) $_n$, in which three peptides with sequence GCA(GPA) $_{14}$ GCA
were cross-linked by a lysyl-lysyl aminohexanoate structure, was
found to have a melting temperature of 29°C. Both showed
insignificant platelet adhesion to monomeric when the peptide
was immobilized on plastic, e.g. (GPP) $_n$ 1% \pm 0.4; collagen 17% \pm
30 1.3. The following results were obtained for platelet
aggregation by crosslinked peptides:

(GPP) $_n$ - active at 10 μ g/ml when CRP active at 50 ng/ml
(i.e. 2,000 times less active than CRP)

35

(GPA) $_n$ - no activity at 1.5 mg/ml when CRP active at 100
ng/ml (i.e. at least 15,000 times less active than CRP).

(GPR)n - also not active up to measurement at 2 mg/ml.

The $\alpha 2\beta 1$ recognition sequence embraced by these peptides, identified as being contained by the collagen-derived sequence from the overlap region of peptides 5 and 6, re-synthesised as Table 1 peptide 5/6, was also synthesised within GPP N-terminal and C-terminal sequences, as an alternative means of stabilising the triple helix. This peptide, lacking GPO sequences, does not react with GpVI and is thus specific for $\alpha 2\beta 1$.

Cell-reactivity of the peptides was investigated in terms of the following properties of these peptides:

1. To support platelet aggregation.
2. To support platelet adhesion.
3. To support the adhesion of the human fibrosarcoma cell line HT1080 (which binds to collagen exclusively through $\alpha 2\beta 1$). (The involvement of $\alpha 2\beta 1$ in 1 and 2 is determined by the sensitivity of adhesion to the inclusion of functionally-blocking antibodies directed against $\alpha 2\beta 1$.)
4. To bind purified platelet $\alpha 2\beta 1$.
5. To bind recombinant I-domain of $\alpha 1$.

METHODS

Platelet adhesion and aggregation

Platelet adhesion was measured in 35mm or other petri dishes (for which Falcon 1008 are suitable) using either a colorimetric method for cell adhesion (see Knight et al 1999) or ^{51}Cr -labelled gel-filtered human platelets as described (Zijenah and Barnes, 1990). When testing mAbs for inhibitory activity, platelets were preincubated with antibody for 15 min. Statistical significance of the effect of mAbs was determined by one-way analysis of variance ("ANOVA"), using readily available computer statistical software (Instat, produced by GraphPad, San Diego, CA, USA), either within experiments or using the mean values from at least three separate experiments.

Platelet aggregation was measured turbidimetrically using

human citrated platelet-rich plasma as previously (Morton et al., 1995). Alternative methods of measuring aggregation are possible, e.g. electronic particle counting or impedance measurement (McNicol, 1995).

5

Adhesion of human fibrosarcoma (HT 1080) cells

HT 1080 cells, obtainable for example from the European Collection of Animal Cell Cultures, Porton Down, Wiltshire, UK, were maintained in Eagle's MEM containing 15% FBS, 2mM
10 glutamine, penicillin (100 I.U./ml), streptomycin (100 µg/ml) and amphotericin (2.5 ng/ml), or other growth medium as appropriate. Cells were harvested with trypsin/EDTA, suspended in Eagle's MEM or other buffer containing 20% FBS, washed four times with Dulbecco's PBS (Ca²⁺ and Mg²⁺ free) and finally
15 suspended in adhesion buffer (TBS) containing 1mM Mg²⁺. Immulon 2 or other suitable multi-well plates were coated with collagen or peptide, normally at 10 µg/ml, for 1h at 20 °C. Cell suspension (0.1ml, 3x10⁴ cells) was added to each well and adhesion measured after 90 min at 20 °C or 37 °C, as required.
20 Adhesion was determined using a Coulter Counter (model ZF) to count unattached cells. The colorimetric method used for platelets (Bellavite et al., 1994 Anal. Biochem. 216: 444-4500) was readily adapted to provide a suitable alternative for HT1080 and other cell adhesion assays. Cells were preincubated with
25 antibody, when testing for inhibition, for 15 min. The significance of any inhibition of adhesion by mAbs was tested using the same statistical analysis as for platelets.

Isolation of integrin $\alpha 2\beta 1$

30 Integrin $\alpha 2\beta 1$ was purified from solubilized membranes of human platelets by affinity chromatography on collagen-Sepharose as described in detail by Messent et al. (Kern et al., 1993). The purity was established by polyacrylamide gel electrophoresis, immunoprecipitation and Western blotting.
35 Protein concentration was determined with BCA reagent (Pierce) or other suitable methodology and the purified integrin biotinylated using a biotinylation kit (available for example from Amersham) according to the manufacturer's instructions.

Integrin $\alpha 2 \beta 1$ binding assay

The assay procedure was essentially as described by Tuckwell et al., 1995. Briefly, wells of 96-well ELISA plates (Nunc Maxisorp) were coated with 100 μ l of a solution of collagen or peptide, 1-10 μ g/ml in 0.01M-acetic acid for 2h at room temperature. Wells were then blocked for 1h with 100 μ l of TBS (150mM NaCl in 50mM tris-HCl, pH 7.4) containing BSA (Sigma, grade A4503) at 50mg/ml and washed three times with 200 μ l of TBS containing BSA (Sigma, grade A7638) at 1mg/ml (washing buffer). 100 μ l of adhesion buffer (washing buffer plus either 2mM-MgCl₂ and 1mM-MnCl₂ or 10mM-EDTA [disodium salt], as required) containing biotinylated integrin at 0.5 μ g/ml, was applied to each well and incubated for 2h at room temperature. Wells were then washed as above and incubated for 30min with 100 μ l of streptavidin-HRP (Amersham Life Sciences) diluted 1:1500 with washing buffer. The wells were again washed three times and bound integrin detected using a TMB-peroxidase substrate system (KPL, Gaithersburg, Maryland, USA) according to the manufacturer's instructions. Optical density was recorded using a Maxline Emax plate reader (Molecular Devices) or other suitable instrument. Assays were undertaken in triplicate and values were corrected for a background reading obtained by coating wells with 0.01M-acetic acid in the absence of peptide or collagen substrate.

Production of recombinant integrin $\alpha 1$ and $\alpha 2$ I-domains

Recombinant human $\alpha 1$ and $\alpha 2$ integrin I-domains were produced, as described in detail by Tuckwell et al., 1995, from the pertinent cDNA generated by reverse transcription-polymerase chain reaction and cloned into pGEX-2T for transformation of E. coli strain DH5 α F'. Integrin $\alpha 1$ or $\alpha 2$ I-domain-glutathione-S-transferase (GST) fusion protein was isolated from bacterial lysates by affinity chromatography on a glutathione-agarose column.

Integrin $\alpha 1$ -domain or $\alpha 2$ I-domain binding assay

Binding was assayed essentially as described by Tuckwell et al., 1995. Briefly, Nunc Maxisorp multiwell plates were coated

for 1h at room temperature with collagen or peptides and blocked with BSA, as described above. After blocking, wells were washed three times with TBS containing 1mM-MgCl₂ and BSA (1mg/ml). 100 μ l of a solution of α 1 or α 2 I-domain fusion protein (or
5 thrombin-cleaved I-domain, which served interchangeably) at 50 μ g/ml in the above buffer or one containing 5mM-EDTA in place of Mg²⁺, was applied to wells and plates incubated for 3h at room temperature. Wells were then washed as before. Bound I-domain was detected by the addition of 100 μ l per well of polyclonal
10 rabbit anti-GST, 10 μ g/ml in TBS (plus 1mM-MgCl₂ and BSA, 1mg/ml). Plates were incubated for 45min and then washed three times as above. 100 μ l of peroxidase-conjugated goat anti-rabbit IgG (DAKO) diluted 1:2000 in TBS (plus Mg²⁺ and BSA) was added to wells and plates incubated for a further 45min, then
15 given a final wash as above. Wells were then treated with a TMB-peroxidase substrate system (KPL) and the colour read at 450nm using the Emax plate reader. Assays were undertaken in triplicate and readings corrected for background as above.

20 Peptide synthesis

Any suitable method of synthesising peptides may be used. The following was found to be suitable.

Peptides were synthesized as C-terminal amides on TentaGel R RAM resin in a PerSeptive Biosystems 9050 Plus PepSynthesiser
25 by Fmoc/t-Bu chemistry. Side chain protecting groups were as follows: Arg(Pbf), Asn(Trt), Asp(Ot-Bu), Cys(Trt), Gln(Trt), Glu(Ot-Bu), Hyp(t-Bu) and Lys(Boc). In general, Fmoc-amino acids (4 eq.) were activated with HATU (4 eq.) in the presence of diisopropylethylamine (8 eq.) (Carpino et al., (1994) *J. Chem. Soc, Chem. Commun.* 201-203). HOAt (4 eq.) was added when
30 coupling Asn and Gln. Fmoc deprotection was with a mixture of 2% (v/v) piperidine and 2% (v/v) 1,8-diazabicyclo[5,4,0]undec-7-ene, except with peptides containing Asp when 20% piperidine and 0.1M 1-hydroxbenzotriazole in dimethylformamide were used to
35 minimize aspartimide formation (Martinez, J. and Bodanszky, M. (1978) *Int. J. Pept. Prot. Res.* 12, 277; Fields et al., (1996) *Lett. Pept. Sci.* 3, 3-16). Peptides containing Asp-Gly sequences are especially prone to give aspartimide (Quibell et

al., (1994) *J. Chem. Soc., Chem. Commun.* 2343-2344) and these were made using Fmoc-(Fmoc-Hmb)-Gly and Fmoc-Asp(Ot-Bu)-OPfp (Packman, L. C. (1995) *Tetrahedron Lett.* 36, 7523-7526).

Peptides were released from the resin by treatment with a mixture of trifluoroacetic acid, thioanisole, ethanediol and triisopropylsilane (90:5:2.5:2.5, by vol.) for 6 hours at room temperature. Crude peptides were purified by reverse phase HPLC on a column of Vydac 219TP101522 using a linear gradient of 5 to 45% acetonitrile in water containing 0.1% trifluoroacetic acid. Fractions containing homogeneous product were identified by analytical HPLC on a column of Vydac 219TP54, pooled and freeze-dried. The identity of the purified peptides was confirmed by mass spectrometry.

In all, seven overlapping peptides, designated 1-7, based on the sequence of the fragment 1(I)CB3, were synthesized (see Table 1). In addition, several related peptides were made, based upon the overlap sequence between peptides 5 and 6, substituting particular amino acids or deleting specific amino acids. The sequences are shown in Tables 1A to 1D. Flanking sequences of either GPO or GPP triplets were used to maintain triple helical structure, as shown in Tables 1A to 1D.

The triple-helical stability of each peptide was assessed by polarimetry as previously described.

Crosslinking

Peptides were crosslinked with 3-(2-pyridyldithio)-propionic acid N-hydroxysuccinimide ester as before (Morton et al., 1995).

RESULTS

It is important that peptides have a conformationally constrained structure for interaction with their receptors. This was verified for the peptides of triple-helical conformation, by optical rotation using a polarimeter (Morton et al., 1995).

Specimen results for Peptide 6 are shown in Figure 1.

Melting temperatures for the peptides were as follows:

	Peptide 1	32°C
	Peptide 2	39°C
	Peptide 3	37°C
5	Peptide 4	36°C
	Peptide 5	30°C
	Peptide 6	30°C
	Peptide 7	26°C
	Peptide 5/6	39°C
10	Des-E 5/6	44°C

Where sequences were synthesised within GPP, GPA or GPR N- and C-terminal polymers, melting temperatures were characteristically lower, but were greater than room temperature, allowing these peptides to be tested in triple-helical conformation. The sequence GFOGER was also synthesised within GAP polymeric N- and C-terminal peptides. No melting curve could be obtained for this peptide, indicating that it did not adopt triple-helical structure.

Figure 2 shows that intact collagen binds the $\alpha 2\beta 1$ integrin, whereas Peptides 1, 2, 3 & 4 do not. Peptide 7 was also found not to bind, as shown in Figure 3. Peptides 5 and 6 bound collagen with greater avidity than collagen, as is shown in Figure 3. The $\alpha 2\beta 1$ integrin recognition site was localised in the overlap region of Peptides 5 and 6, corresponding to the sequence GFOGERGVEGPOGPA. Peptide 5/6 was synthesized with this sequence.

HT1080 cells express $\alpha 2\beta 1$ integrin and so were used in a further test of adhesion mediated by this integrin. The cells showed no significant adhesion to Peptides 2, 3, 4 and 7, but good adhesion to Peptides 5, 6 and 5/6 - as good as to collagen, i.e. about 80% at room temperature at 90 minutes. Adhesion to Peptide 1 was about 60%.

Adhesion to collagen and Peptides 1, 5, 6 and 5/6 was strongly inhibited by anti- $\alpha 2$ integrin, 6F1 (Coller et al., 1989, *Blood*, 74:182-192) inhibition varying between experiments from 65-90%. Any residual adhesion in the presence of 6F1 occurred in the total absence of cell

spreading.

Adhesion of recombinant $\alpha 2$ I-domain to collagen and to peptide 5/6, also the 5/6GEA and 5/6GAR variants, was assayed. The results are shown in Figure 4. It was found
5 that adhesion was inhibited by EDTA, a chelator which binds Mg^{2+} , needed for binding activity of the integrin, thus verifying the assay. The Peptide 5/6GAR was found to lack capacity to bind $\alpha 2\beta 1$, indicating the importance of Glu residues for binding of 5/6 (and by inference collagen) to
10 $\alpha 2\beta 1$, and similarly, the Arg residue could not be substituted by Ala.

Adhesion of both $\alpha 2$ I-domain and $\alpha 2\beta 1$ to Peptide 5/6 was also inhibited by the 6F1 antibody and by EDTA. Similar data were obtained using HT1080 cells and intact platelets.

15 Figures 5 and 6 show the effect of deleting amino acid triplets from the 5/6 overlap peptide. Adhesion of the $\alpha 2$ I-domain (and of platelets, HT1080 cells and purified $\alpha 2\beta 1$, not shown) was retained by peptides Des-GPA, Des-GVE and Des-GPO, but Des-GFO did not support adhesion, indicating that the
20 sequence GFOGER contained the crucial determinants of reactivity with $\alpha 2$ I-domain, and with the intact integrin.

Figure 7 shows the importance of the Glu (E) residue within the GFOGER sequence, since the peptide GFOGDR (synthesised within GPP polymeric structures, was unable to
25 support $\alpha 2\beta 1$ binding.

Figure 8 shows that this same sequence, GFOGER, in Des-GVE, supports binding of the $\alpha 1$ I-domain, so that the sequence is also recognised by the integrin $\alpha 1\beta 1$. GFOGDR showed no ability to support $\alpha 1$ binding.

30 Figure 9 shows that substitution of R by K, in the peptide GFOGEK, does not support $\alpha 2$ binding. Hence two conservative substitutions may destroy the ability of the sequence to recognise $\alpha 2\beta 1$. This was verified using purified $\alpha 2\beta 1$ binding and intact platelet binding.

35 The peptide MADE42 comprises tandem GFOGER motifs separated by intervening GPP triplets, and with GPP triplets at the N and C-termini of the peptide. This peptide showed adhesion of $\alpha 2$ I-domain as good as type I collagen, although

rather lower than Des-GVE-GPP which contains a single copy of the GFOGER motif. This peptide will provide a tool to study crosslinking of receptors such as $\alpha 2\beta 1$ and $\alpha 1\beta 1$.

5 The use of peptides to block cell binding to collagen, is an important part of the present invention. Platelets were incubated with Des-GVE-GPP, and then allowed to interact with collagens I and IV. Inhibition of the interaction is shown in Figure 10, using both Type I and Type IV collagens as substrates.

10 Similarly, binding of $\alpha 1$ and $\alpha 2$ I-domains to collagen could be inhibited by increasing levels of Des-GVE-GPP, indicating that this same sequence can provide blockade of both $\alpha 1\beta 1$ and $\alpha 2\beta 1$. These data are shown in Figure 11.

15 Figure 12 shows the importance of peptide conformation in this respect: a version of the sequence GFOGER was synthesised in GAP polymeric sequences, which do not support triple helical structure, i.e. no melting curve as in Figure 1 could be obtained. This peptide, Des-GVE-GAP, did not inhibit $\alpha 2$ I-domain binding.

20 Figure 13 shows firstly, that GPP polymers do not support platelet adhesion, although CRP, the GPO polymer does so. Two variant peptides were synthesised and tested, AcGPO2 and AcGPO4. These are essentially GPP polymers containing tandem and quadruplet GPO sequences, as shown in Table 1.
25 The data indicate that only partial adhesion is obtained using AcGPO2 compared with CRP, whereas AcGPO4 supported a level of adhesion lower, though not significantly so, than CRP. This indicates that the ligand for GpVI may consist of a longer peptide than a single GPO motif, or even two such
30 sequences in tandem. It is clear that full binding of triple helical peptides to GpVI may require longer molecules than the $\alpha 2\beta 1$ -binding motif GFOGER described above.

Figure 14 provides proof, obtained using the anti-GpVI Fab fragment, that adhesion of platelets to AcGPO4 and to
35 CRP, though not collagen, is dependent upon GpVI, but is independent of $\alpha 2\beta 1$, since the anti- $\alpha 2$ antibody, 6F1 had little effect on platelet adhesion. In turn, this provides proof, in conjunction with Figure 13, that neither the GPO

not GPP motifs recognise $\alpha 2\beta 1$.

Figure 15 indicates interchangeability of hydroxyproline (O or P*) and proline (P) residues in recognition by $\alpha 2\beta 1$. Peptides containing variants of the 15-amino acid sequence from the overlap region of Peptides 5 and 6 (Table 1A) were used as substrates for adhesion of $\alpha 2$ I-domain. Peptide GPP-56 contains two proline residues in place of hydroxyproline, and supports good adhesion. Peptide 56-Hyp contains proline only in the first triplet of the sequence, and supports rather better adhesion. Substitution of P for O with GFOGER permits retention of $\alpha 2\beta 1$ -binding. Substituting alanine (A) for both glutamates (E) as well as P for O (Peptide Des-E-GPP) results in loss of adhesive capacity for $\alpha 2\beta 1$.

TABLE 1A. PEPTIDES IN GPO N- AND C-TERMINAL SEQUENCES

	PEPTIDE 1
5	GPC (GPO) ₃ GFOGPKGAAGEOGKAGERGVOGPOGAVGPA (GPO) ₃ GPC
	PEPTIDE 2
	GPC (GPO) ₃ GPOGAVGPAGKDGEAGAQQGPOGPAGPAGER (GPO) ₃ GPC
10	PEPTIDE 3
	GPC (GPO) ₃ GPAGPAGERGEQQGPAGSOGFQGLOGPAGPO (GPO) ₃ GPC
	PEPTIDE 4
	GPC (GPO) ₃ GLOGPAGPOGEAGKOGEGQVGVDLGAOGPS (GPO) ₃ GPC
15	PEPTIDE 5
	GPC (GPO) ₃ GDLGAOGPSGARGERGFOGERGVGPOGPA (GPO) ₃ GPC
	PEPTIDE 6
20	GPC (GPO) ₃ GFOGERGVGPOGPAGPRGANGAOGNDGAK (GPO) ₃ GPC
	PEPTIDE 7
	GPC (GPO) ₃ GANGAOGNDGAKGDAGAOGAOGSQGAOGLQ (GPO) ₃ GPC
	PEPTIDE 5/6
25	GPC (GPO) ₃ GFOGERGVGPOGPA (GPO) ₃ GPC

Table 1B. Variant Peptides based on 5/6

PEPTIDE Des-E 5/6

GPC (GPO)₃ GFOGARGVAGPOGPA (GPO)₃ GPC

5

PEPTIDE 5/6GAR (DesE 5/6)

GPC (GPO)₃ GFOGARGVEGPOGPA (GPO)₃ GPC

PEPTIDE 5/6GEA

10 GPC (GPO)₃ GFOGEAGVEGPOGPA (GPO)₃ GPC

PEPTIDE Des-GPA

GPC (GPO)₃ GFOGERGVGPO (GPO)₃ GPCG

15 PEPTIDE Des-GFO

GPC (GPO)₃ GERGVGPOGPA (GPO)₃ GPCG

PEPTIDE Des-GPO

GPC (GPO)₃ GFOGERGVE (GPO)₃ GPCG

20

PEPTIDE Des-GVE

GPC (GPO)₃ GFOGER (GPO)₃ GPCG

TABLE 1C. PEPTIDES IN GPP N- AND C-TERMINAL SEQUENCES

	PEPTIDE GPP 56
	GPC(GPP) ₅ GFPGERGVGGPPGPA(GPP) ₅ GPC
5	
	PEPTIDE DesE-GPP
	GPC(GPP) ₅ GFPGARGVAGPPGPA(GPP) ₅ GPC
	PEPTIDE 56-Hyp
10	GPC(GPP) ₅ GFPGERGVGGPOGPA(GPP) ₅ GPC
	PEPTIDE Des-GVE-GPP
	GPC(GPP) ₅ GFOGER (GPP) ₅ GPC
15	PEPTIDE GFOGDR
	GPC(GPP) ₅ GFOGDR (GPP) ₅ GPC
	PEPTIDE GFOGEK
	GPC(GPP) ₅ GFOGEK (GPP) ₅ GPC
20	
	PEPTIDE MADE 42
	GPC(GPP) ₃ GFOGER (GPP) ₄ GFOGER (GPP) ₃ GPC

TABLE 1D.

OTHER PEPTIDE SEQUENCES SUPPORTING TRIPLE HELICAL STRUCTURE

	PEPTIDE Des-GVE-GAP
5	GPC (GAP) ₅ GFOGER (GAP) ₅ GPC
	PEPTIDE [GPP] _n
	GCP (GPP) ₁₀ GCPG
10	Collagen-Related Peptide (CRP)
	GCO (GPO) ₁₀ GCOG
	PEPTIDE [GPR] _N
	GCP (GPR) ₁₀ GCPG
15	PEPTIDE [GPA] _n
	GCA (GPA) ₁₄ GCA-aminohexanoic acid
	(covalent trimer as described in Morton et al 1997)
20	Peptide AcGPO2
	Ac-GCO [GPP] ₄ [GPO] ₂ [GPP] ₄ GCPG-NH ₂
	Peptide AcGPO4
	Ac-GCO [GPP] ₃ [GPO] ₄ [GPP] ₃ GCPG-NH ₂
25	(These last two peptides are acetylated at the N-terminus and have amide groups at the C-terminus.)

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CLAIMS:

1. A peptide consisting of a sequence of amino acids selected from the following:
- 5 (i) GFOGERGVEGPOGPA (SEQ ID NO. 1),
 (ii) GFPGERGVEGPPGPA (SEQ ID NO. 2),
 (iii) a fragment of (i) or (ii) which retains ability to interact with and/or bind integrin $\alpha 2\beta 1$.
- 10 2. A peptide according to claim 1 which consists of a minimal sequence of amino acids which retains ability to interact with and/or bind integrin $\alpha 2\beta 1$.
- 15 3. A peptide consisting of an amino acid sequence variant of a peptide according to claim 1, which amino acid sequence variant consists of 10-15 contiguous amino acids wherein at least 10 amino acid residues match corresponding amino acid residues in SEQ ID NO. 1 or SEQ ID NO. 2.
- 20 4. A peptide consisting of an amino acid sequence variant of a peptide according to claim 1, which amino acid sequence variant consists of an amino acid sequence according to the following formula:
 G X₁ O/P G X₂ X₃
- 25 wherein X₁, X₂ and X₃ represent any amino acid.
5. A peptide according to claim 4 wherein X₁ is F.
6. A peptide according to claim 4 or claim 5 wherein X₂ is
30 E or D.
7. A peptide according to any one of claims 4 to 6 wherein X₃ is R or K.
- 35 8. A peptide according to claim 7 which consists of an amino acid sequence selected from:
 GFOGER
 GFPGER

GFPGEK
GFPGDK
GFPGDR.

- 5 9. A peptide consisting of a peptide according to any one
 of the preceding claims and (GPP) repeats.
10. A peptide consisting of a peptide according to any one
 of claims 1 to 8 and (GPO) repeats.
- 10 11. A peptide consisting of a peptide according to any one
 of claims 1 to 8 flanked by (GPP) repeats.
12. A peptide consisting of a peptide according to any one
15 of claims 1 to 8 flanked by (GPO) repeats.
13. A peptide according to any one of the preceding claims
 fused to heterologous amino acids.
- 20 14. A peptide trimer comprising a peptide according to any
 one of the preceding claims.
15. A peptide trimer according to claim 14 consisting of
 three peptides according to any one of claims 1 to 13.
- 25 16. A peptide trimer according to claim 15 which is a
 homotrimer.
17. A method of making a peptide trimer according to any one
30 of claims 14 to 16, the method comprising a step of bringing
 together under conditions for formation of a peptide trimer
 three peptides which form a peptide trimer, at least one of
 said three peptides being a peptide according to any one of
 claims 1 to 8.
- 35 18. A method according to claim 17 wherein said step is
 preceded by production of at least one of said three peptides
 by expression from encoding nucleic acid, and optionally

25. A method of obtaining a mimetic of a peptide according to any one of claims 1 to 13, the method comprising:

- 5 (i) analysing a said peptide to determine the amino acid residues essential and important for ability to interact with $\alpha 2\beta 1$ receptor to define a pharmacophore; and,
- (ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

26. Use of a peptide according to any one of claims 1 to 13
10 in a method of designing or screening for a mimetic of the peptide.

hydroxylating one or more proline residues to provide hydroxyproline (O).

19. An isolated nucleic acid encoding a peptide according to any one of claims 1 to 13.

20. A method of making a desired peptide according to any one of claims 1 to 13, the method comprising causing expression from nucleic acid according to claim 19 to produce an encoded peptide, and optionally, where the desired peptide includes one or more hydroxyproline (O) residues, hydroxylating one or more proline residues in the encoded peptide to provide said desired peptide.

21. An assay method for obtaining an inhibitor of collagen interaction with either or both of integrin $\alpha 2\beta 1$ receptor and platelet receptor GpVI, the method comprising bringing into contact (i) a peptide according to any one of claims 1 to 13 and (ii) either or both of integrin $\alpha 2\beta 1$ receptor and platelet receptor GpVI, and (iii) a test substance, under conditions wherein in the absence of the test substance being an inhibitor of interaction between the peptide and the receptor or receptors the peptide interacts with the receptor or receptors, and determining interaction between the peptide and the receptor or receptors.

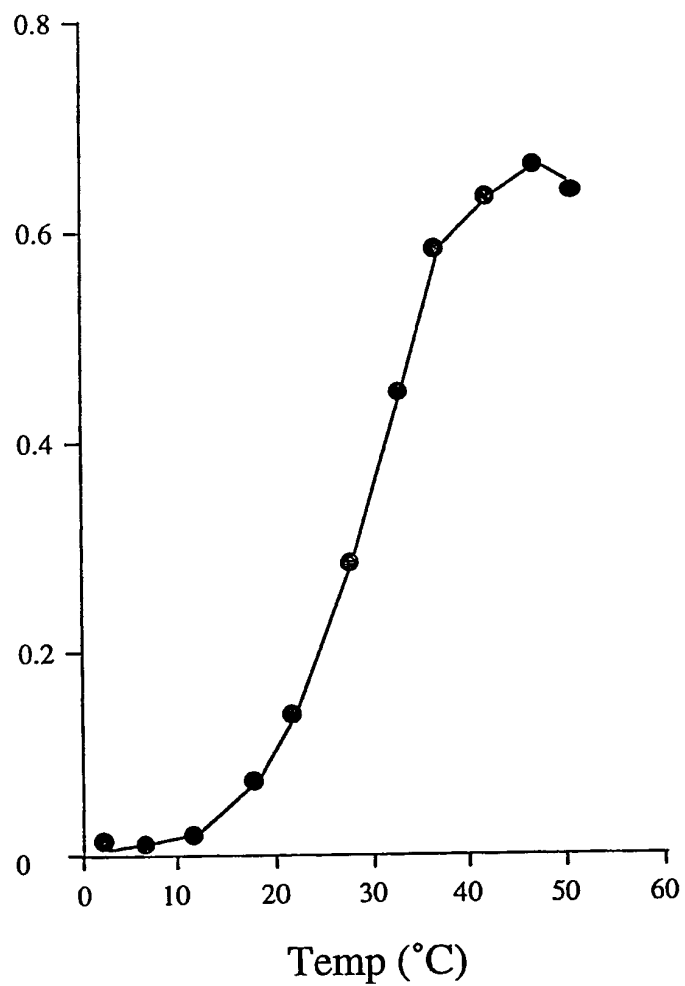
22. An assay method for obtaining an agent which binds a peptide according to any one of claims 1 to 13, the method comprising bringing into contact a peptide according to any one of claims 1 to 8 and a test substance, and determining interaction between the peptide and the test substance.

23. A method according to claim 21 or claim 22 further comprising isolating a said inhibitor or a said agent.

24. A method according to claim 21 or claim 22 further comprising formulating a said inhibitor or a said agent into a composition comprising at least one additional component.

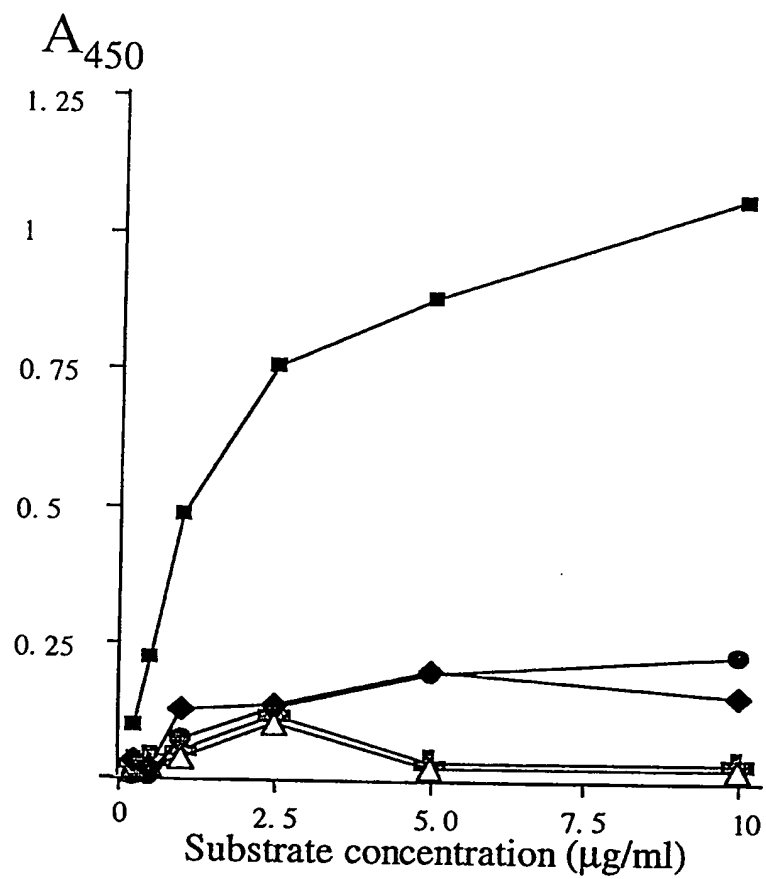
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FIGURE 1



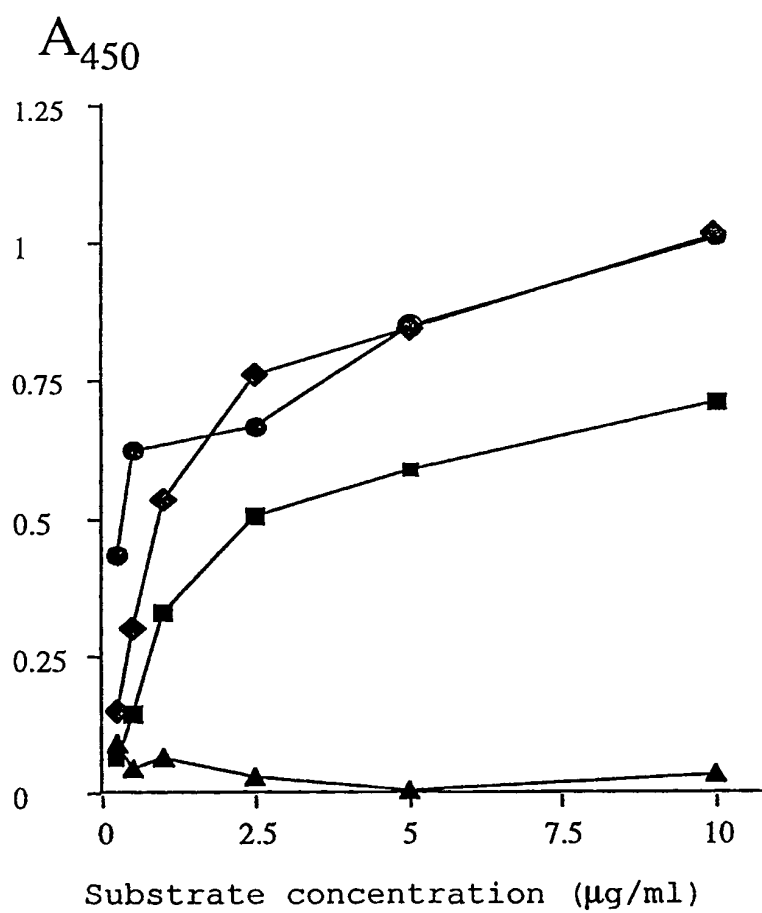
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FIGURE 2



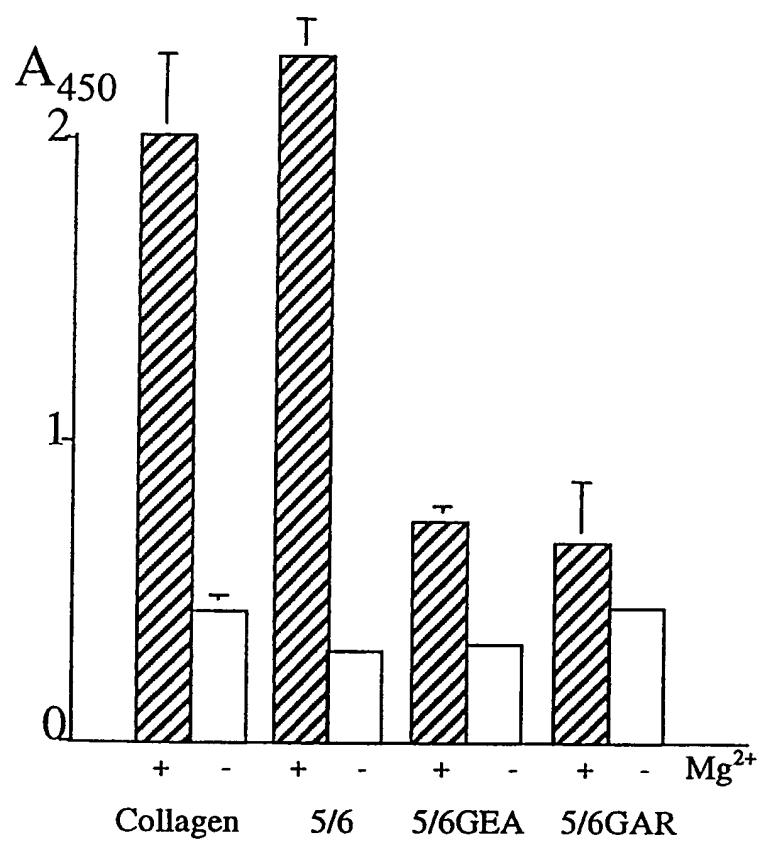
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FIGURE 3



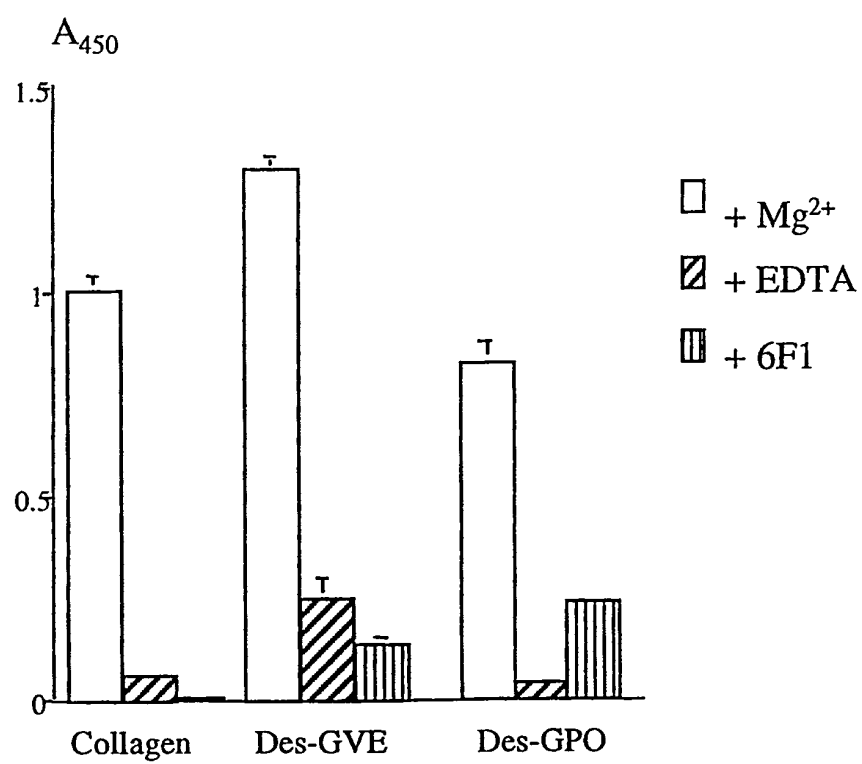
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FIGURE 4



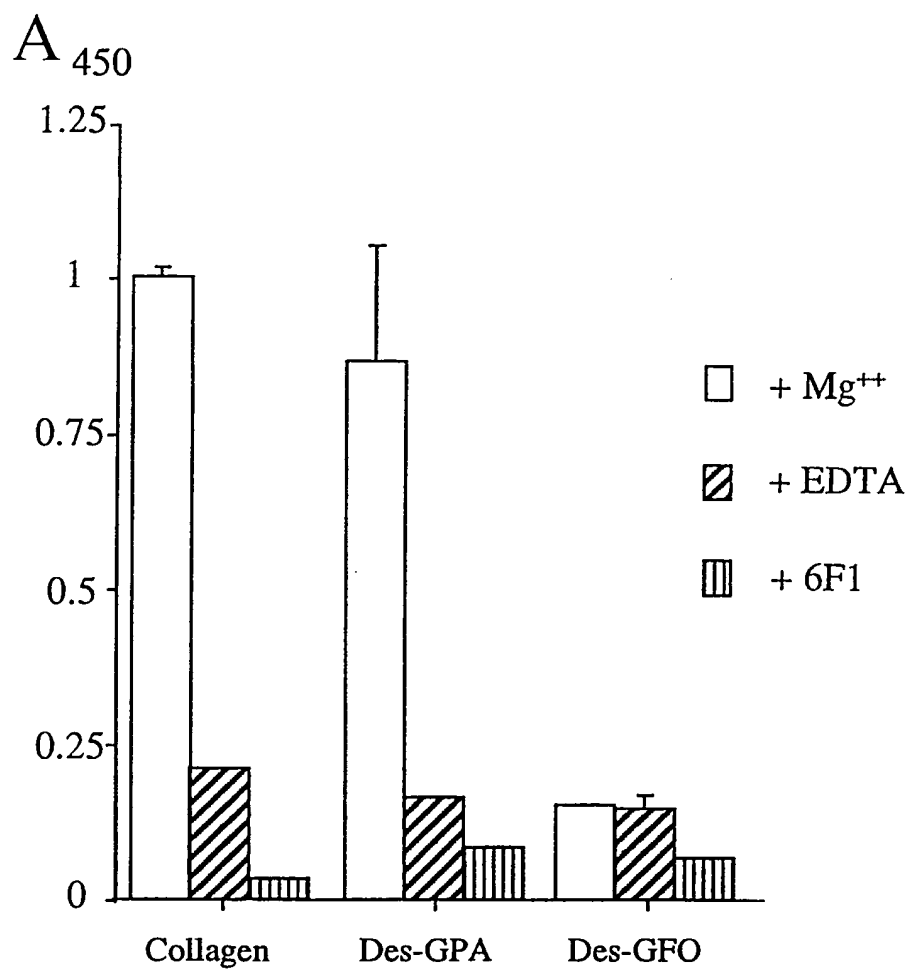
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FIGURE 5



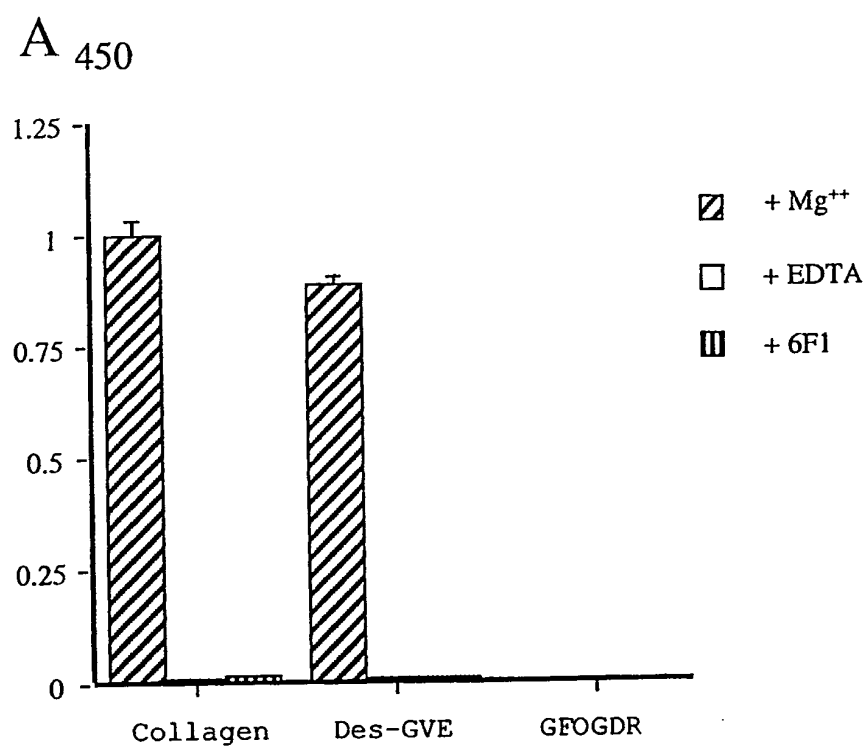
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FIGURE 6



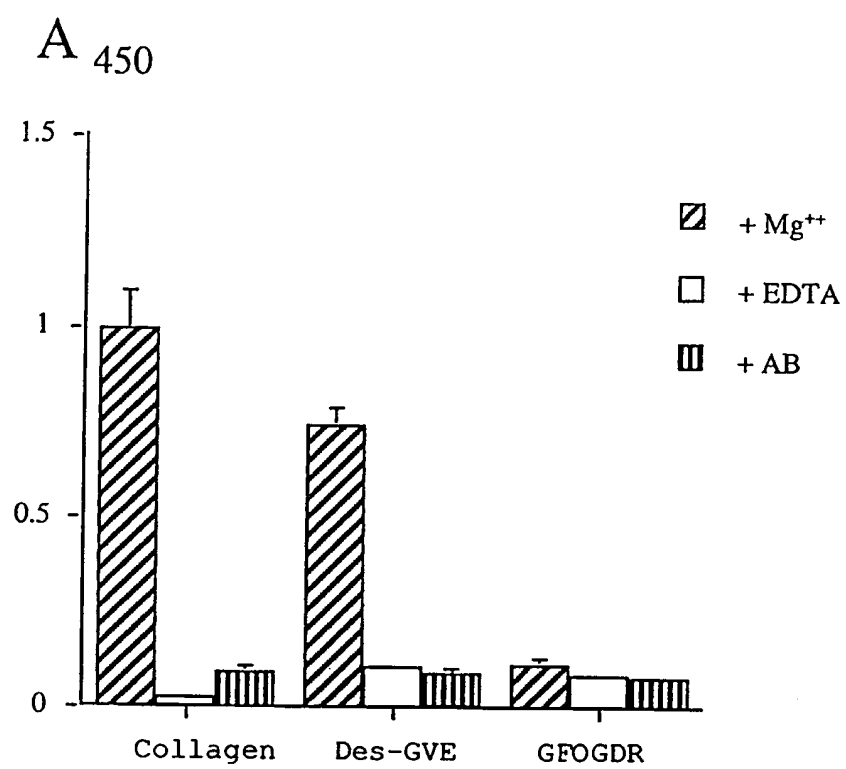
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FIGURE 7



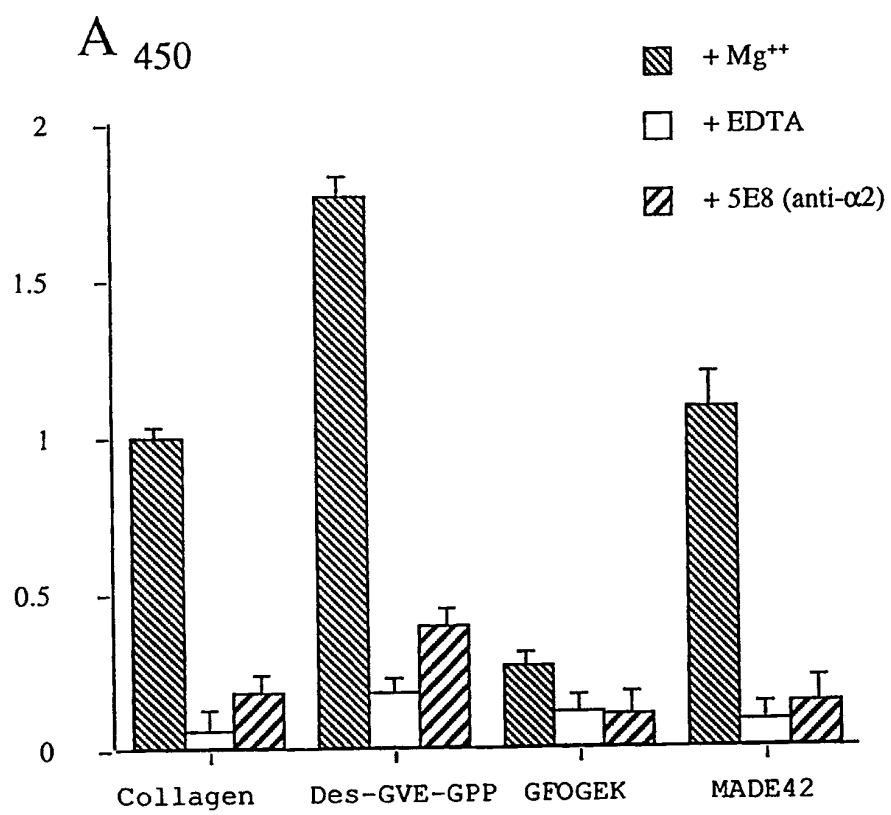
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FIGURE 8



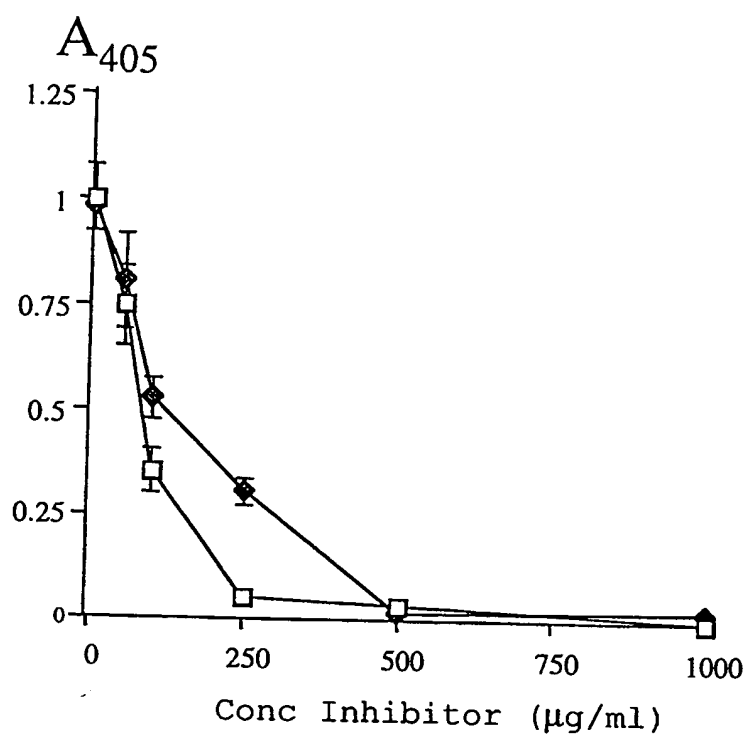
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FIGURE 9



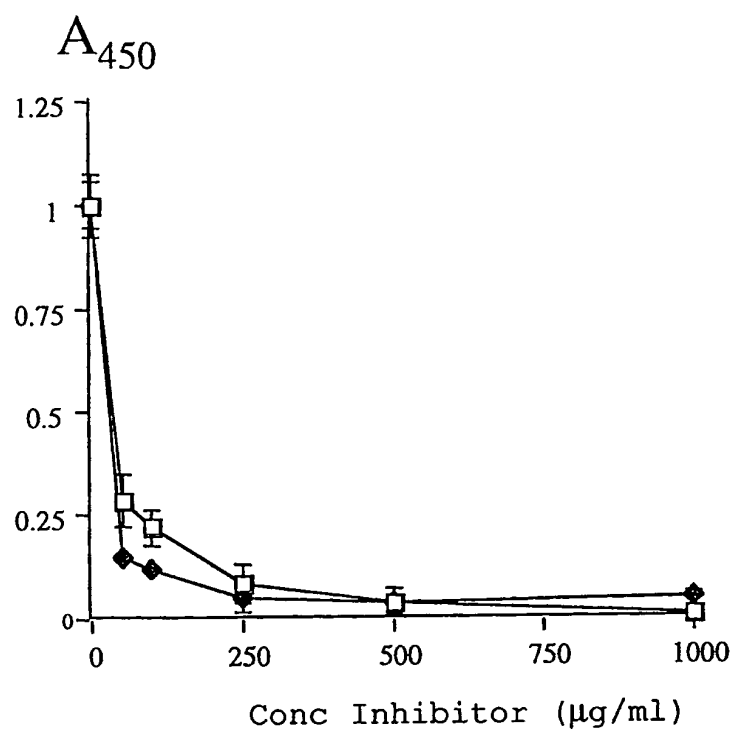
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FIGURE 10



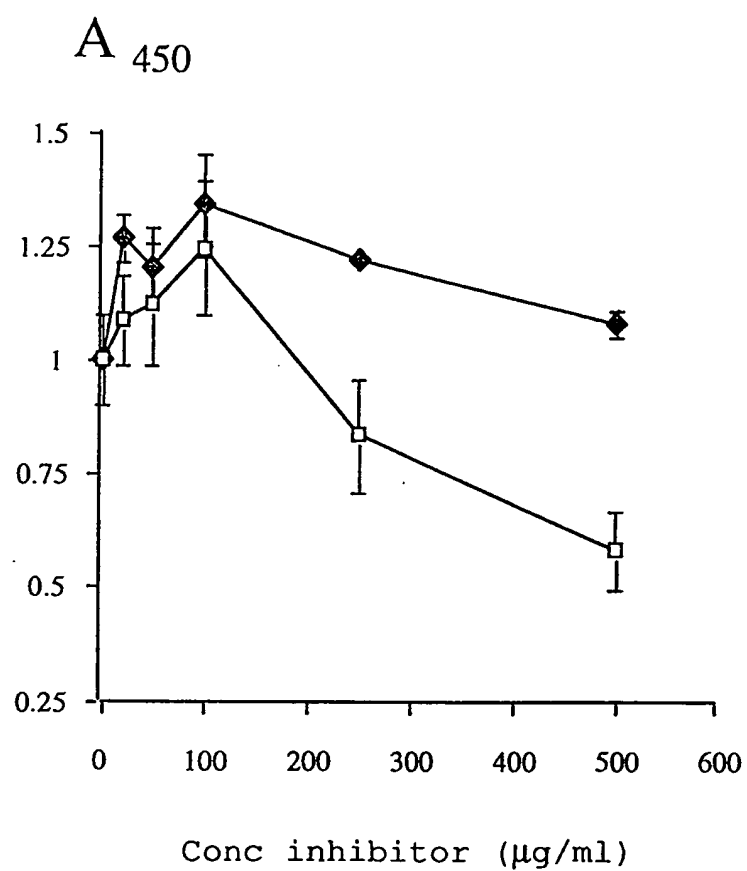
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FIGURE 11



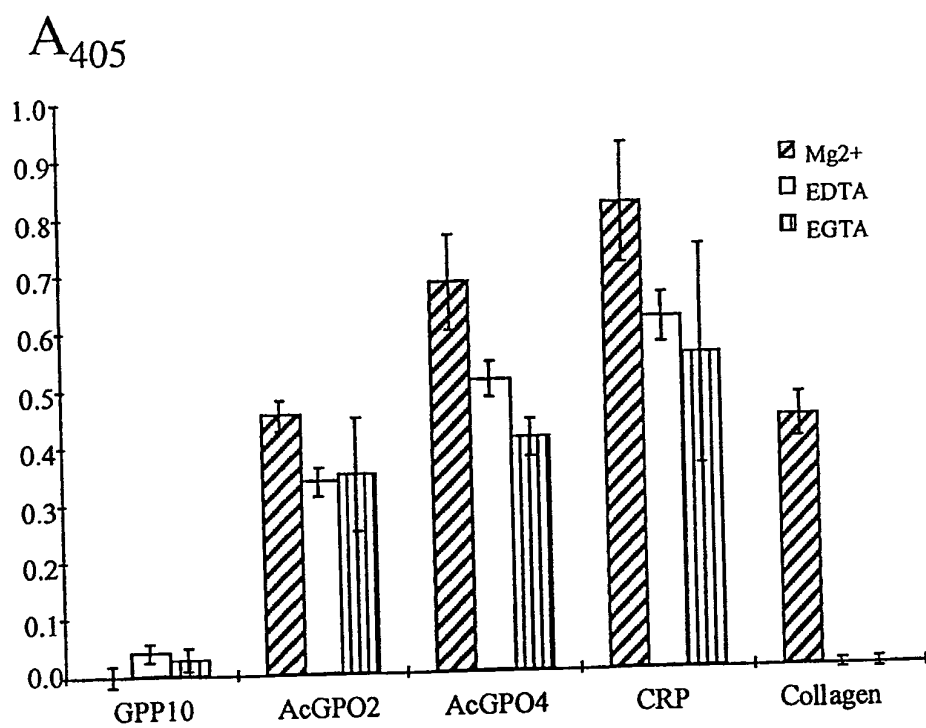
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FIGURE 12



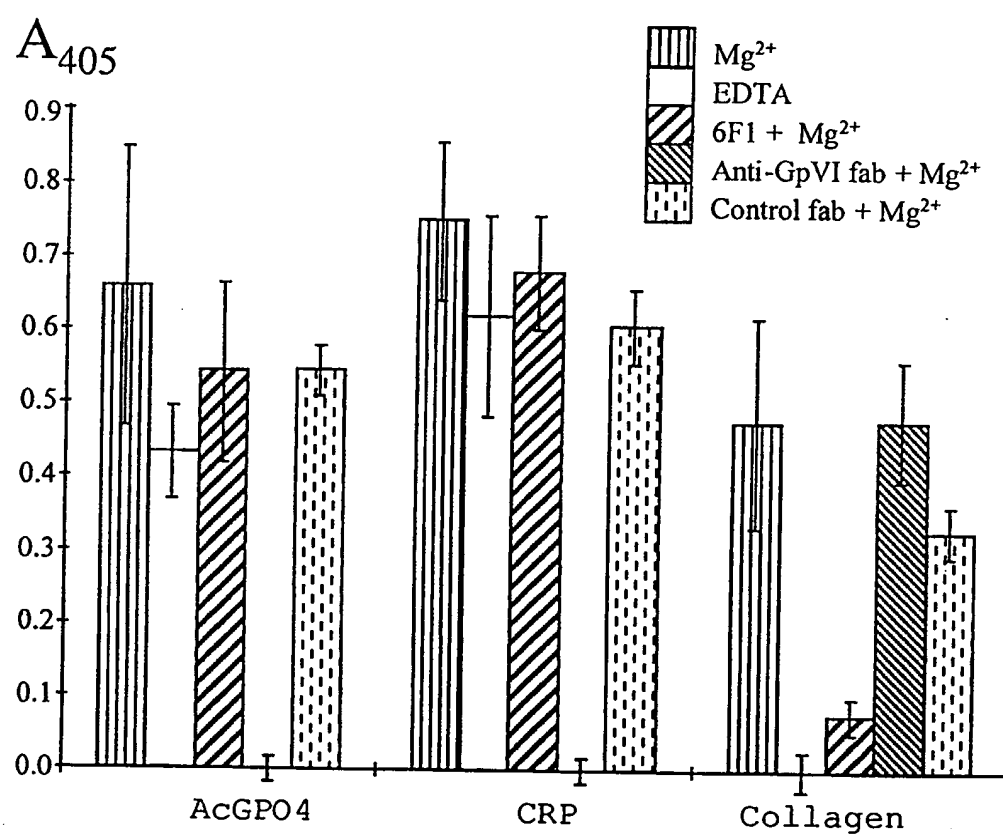
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FIGURE 13



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FIGURE 14



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FIGURE 15

